

## Stable Carbon Isotope Fractionation by *Methanosarcina barkeri* during Methanogenesis from Acetate, Methanol, or Carbon Dioxide-Hydrogen

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*Methanosarcina barkeri* was cultured on methanol, H<sub>2</sub>-CO<sub>2</sub>, and acetate, and the <sup>13</sup>C/<sup>12</sup>C ratios of the substrates and the methane produced from them were determined. The discrimination against <sup>13</sup>C in methane relative to substrate decreased in the order methanol > CO<sub>2</sub> > acetate. The isotopic fractionation for methane derived from acetate was only one-third of that observed with methanol as the substrate. The data presented indicate that the last enzyme of methanogenesis, methylreductase, is not the primary site of isotopic discrimination during methanogenesis from methanol or CO<sub>2</sub>. These results also support biogeochemical interpretations that gas produced in environments in which acetate is the primary methane precursor will have higher <sup>13</sup>C/<sup>12</sup>C ratios than those from environments where other substrates predominate.

Of the two stable isotopes of carbon, <sup>12</sup>C and <sup>13</sup>C, the former is by far the predominant one, constituting 99% of terrestrial carbon. Organic materials display variations in the ratio of <sup>13</sup>C to <sup>12</sup>C, since enzymatic systems often incorporate <sup>12</sup>C preferentially into products. Methane has the largest known variation of the <sup>13</sup>C/<sup>12</sup>C ratio of any hydrocarbon, with reported  $\delta^{13}\text{C}$  values ranging from -13 to -90‰ on the Pee Dee Belemnite (PDB) Carbonate scale (6). The early experiments of Rosenfeld and Silverman demonstrated that a mixed microbial population produced methane that was significantly depleted in <sup>13</sup>C relative to the substrate methanol (18). Later work demonstrated that methane produced from sewage digestors or biodegradation of organic matter in marshes was also very <sup>13</sup>C depleted, with  $\Delta\delta^{13}\text{C}$  values of -47 to -89‰ (2, 16). Methane produced from H<sub>2</sub> and CO<sub>2</sub> by pure cultures of several methanogenic species was substantially depleted in <sup>13</sup>C relative to the substrate (1, 7). On the basis of such studies, it is generally thought that naturally occurring methane that is highly depleted in <sup>13</sup>C (i.e.,  $\delta^{13}\text{C}$  values < -55‰) is of biological origin.  $\delta^{13}\text{C}$  values > -55‰ are usually associated with gases produced by thermal cracking of organic matter during processes such as petroleum maturation. Carbon isotope ratios of methane interpreted in this fashion, in conjunction with other data, are often useful in gas and oil exploration (6).

Acetate is a major precursor of methane in various freshwater environments. These ecosystems release a substantial portion of their methane into the atmosphere (5, 10, 19, 21). In consideration of this, we were interested in determining the amount of discrimination against <sup>13</sup>C displayed by a pure culture of an acetotrophic methanogen common to freshwater sediments. For this purpose, we grew *Methanosarcina barkeri* on three different catabolic substrates (methanol,

carbon dioxide-hydrogen, and acetate) and determined the  $\delta^{13}\text{C}$  values of each of the substrates and of the methane produced from them.

An acetate-adapted culture of *Methanosarcina barkeri*, strain MS, was cultured on the phosphate-buffered basal medium described previously (9, 26). The organism was cultured on 200 mM methanol or sodium acetate in a carboy with 15 liters of phosphate-buffered basal medium and a 5-liter nitrogen headspace. After inoculation with cells grown on the same substrate, the carboy was sparged with nitrogen to remove residual methane. The carboy was incubated at 37°C until the concentration of methane in the gas phase reached approximately 2%, at which time the methane was sampled. Less than 2% of the total substrate had been consumed when the methane sample was taken. Samples of medium were removed at inoculation and at the time methane was sampled. The substrate was isolated from the medium samples as follows. Acetate was separated by steam distillation, neutralized to its sodium salt, and then evaporated to dryness. Methanol was recovered by the collection of distillate produced during heating from 65 to 95°C.

In experiments with H<sub>2</sub>-CO<sub>2</sub> as the growth substrate, a fermentor held at 36°C containing 12 liters of phosphate-buffered basal medium was sparged with 80% H<sub>2</sub>-20% CO<sub>2</sub> at a rate of 1 liter/min. After inoculation with *M. barkeri* MS, cell densities rose to values slightly less than 100 mg/ml, at which time the experiment was ended. Samples of the inlet and outlet gas at the beginning and end of the experiment were collected.

Gas mixtures containing H<sub>2</sub>, CO<sub>2</sub>, and methane were passed through liquid nitrogen traps to remove the CO<sub>2</sub>, which was reserved for isotopic analysis. Methane and methanol samples were converted to CO<sub>2</sub> by combustion at 825°C in a vacuum system modified as described by Craig (3). The total carbon in sodium acetate samples was converted to CO<sub>2</sub> by combustion at 800°C by the Stump and Frazer method (R. K. Stump and J. W. Frazer, Nucl. Sci. Abstr. 28:7848, 1973). The methyl carbon of sodium acetate was converted to CO<sub>2</sub> by pyrolyzing sodium acetate with NaOH at 425°C (15) and then combusting the methane produced as described above. The <sup>13</sup>C/<sup>12</sup>C ratios of CO<sub>2</sub>

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TABLE 1.  $\delta^{13}\text{C}$ -PDB values of substrates and of methane produced from them by *M. barkeri*

Growth substrate	$\delta^{13}\text{C}$ -PDB values (expt 1/expt 2) of <sup>a</sup> :			
	Substrate at $T_0$ <sup>b</sup>	Substrate at $T_F$	$\text{CH}_4$ at $T_F$	$\Delta(\text{CH}_4\text{-substrate})$ <sup>c</sup>
Methanol	-39.9/-39.9	-38.9/-39.5	-114.2/-112.2	-74.8/-72.5
Acetate	-22.2/-22.2 (-17.7/-17.7)	-22.6/-22.0 (-18.4/-17.8)	-43.6/-43.3	-21.2/-21.2
$\text{H}_2\text{-CO}_2$	-10.2/-19.9	-9.6/-18.7	-56.2/-64.5	-46.3/-45.6

<sup>a</sup> Substrate  $\delta^{13}\text{C}$  values are for the methyl carbon; numbers in parentheses are the values for total carbon.  $\delta^{13}\text{C}$  is expressed relative to PDB-carbonate as follows:  $\delta^{13}\text{C}\text{‰} = \{[(^{13}\text{C}/^{12}\text{C})_{\text{sample}}/(^{13}\text{C}/^{12}\text{C})_{\text{standard}}] - 1\} \times 10^3$ .

<sup>b</sup> Substrates at  $T_0$  and  $T_F$  represent the average of inlet and outlet gases. A maximum difference of 1‰ was observed between inlet and outlet  $\delta^{13}\text{C}$  values at  $T_F$ . Data in each column represent the average value of two separate growth experiments.

<sup>c</sup> Calculated by using the average of  $\delta^{13}\text{C}$  values determined from the substrate at  $T_0$  and  $T_F$ . For acetate, the  $\delta^{13}\text{C}$  values of the methyl carbon were used.

samples were analyzed with a mass spectrometer. The precisions on the determinations of  $\delta^{13}\text{C}$  values were  $\pm 0.2\text{‰}$  for  $\text{CO}_2$ , methanol, and the total carbon and the methyl carbon of sodium acetate, and  $\pm 1.0\text{‰}$  for methane. The symbol  $\delta$  represents deviations from the standard PDB-carbonate per thousand.

The results of isotopic analysis of substrates and methane produced from them by *M. barkeri* are shown in Table 1. A wide range of  $\Delta\delta^{13}\text{C}$  values ( $\delta^{13}\text{C}$  methane- $\delta^{13}\text{C}$  substrate) was observed. Methane produced from methanol had  $\delta^{13}\text{C}$  values that were, on the average, 73.6‰ more negative than that of methanol supplied in the medium. A similar result was obtained by Rosenfeld and Silverman (18), who found a  $\Delta\delta^{13}\text{C}$  value of -67.4‰ in their mixed bacterial population. Depletion of  $^{13}\text{C}$  in methane derived from  $\text{CO}_2$  was not as extensive as observed with methanol, the  $\Delta\delta^{13}\text{C}$  values being comparable to those observed for different methanogenic species grown on  $\text{H}_2\text{-CO}_2$  (1, 7). Surprisingly, methane produced from acetate was relatively unenriched in  $^{12}\text{C}$ , with the average  $\Delta\delta^{13}\text{C}$  value half of that observed for carbon dioxide and a third of that observed for methanol. The  $\Delta\delta^{13}\text{C}$  values for acetate were calculated by using the  $\delta^{13}\text{C}$  value of the methyl carbon of acetate, since in this strain of *M. barkeri* 85% of the methane originates from this carbon (12). A similar relationship was found for the  $\Delta\delta^{13}\text{C}$  values of methane produced from methanol or acetate by a mixed microbial population (8, 14).

To date, the biochemical mechanism of isotopic fraction-

ation during methanogenesis is not understood (22, 25, 28-30). However, because our data were obtained from a single organism in pure culture, we can identify some of the reactions responsible for the observed fractionation (Fig. 1). Methyl coenzyme M is the last intermediary metabolite of methanogenesis. Its reduction to methane by the enzyme methylreductase is the common step of methanogenesis from methanol,  $\text{H}_2\text{-CO}_2$ , or acetate (4, 11, 13, 20). The universality of this reaction suggests that methylreductase is not the major site of  $^{13}\text{C}$  discrimination in the formation of biological methane because of the different fractionation values we observed for these three substrates. The  $\Delta\delta^{13}\text{C}$  values for methanogenesis from methanol were more than three times larger than those observed for acetate. Therefore, at least two-thirds of the observed discrimination must occur before the formation of methyl coenzyme M. The two corrinoid enzymes responsible for methanol activation and conversion to methyl coenzyme M have been characterized. These are methyl transferases I and II (23, 24). Our data suggest that the major site of isotopic discrimination during methanogenesis from methanol must be one or both of these enzymes. Likewise, at least half of the observed isotopic discrimination during methanogenesis from  $\text{CO}_2$  must occur during the reduction of  $\text{CO}_2$  to the methyl level. These data also rule out the possibility of the generation of intermediate free methane during methane formation from acetate, since this would lead to similar  $\Delta\delta^{13}\text{C}$  values for the methane produced from the two substrates.

Methane in marine environments arises principally from methanol and methylamine, and not from  $\text{H}_2\text{-CO}_2$  or acetate, because the latter two substrates are preferentially consumed by sulfate-reducing bacteria rather than by methanogens (17). Recently, Whiticar et al. (27) suggested that environments low in sulfate, in which acetate is the primary methane precursor, can be identified by the relatively high  $\delta^{13}\text{C}$  values and low  $\delta\text{D}$  values of the methane produced therein. They proposed that in freshwater environments, methane can be defined isotopically by  $\delta^{13}\text{C}$  values between -60 and -50‰, whereas in marine environments  $\delta^{13}\text{C}$  values are more negative than -60‰. Our results obtained with a pure culture of *M. barkeri* support the contention that methanogenesis from acetate produces less isotopic fractionation than reactions associated with methanogenesis from methanol or  $\text{H}_2\text{-CO}_2$ .

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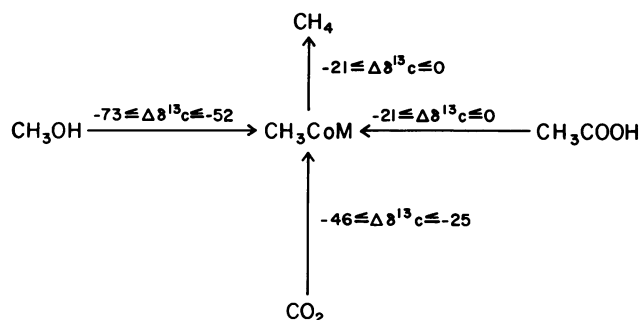


FIG. 1. Schematic of isotopic fractionation during partial reactions of methanogenesis from methanol, acetate, or carbon dioxide-hydrogen. The  $\Delta\delta^{13}\text{C}$  observed during transformation of the methyl group of acetate to methane is taken as the upper limit of discrimination against  $^{13}\text{C}$  during the conversion of methyl coenzyme M ( $\text{CH}_3\text{CoM}$ ) to methane. As a result, the range of  $\Delta\delta^{13}\text{C}$  values for the conversion of each substrate to methyl coenzyme M can be calculated and are presented next to each partial reaction. It is assumed that methylreductase does not discriminate against  $^{12}\text{C}$ .

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